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(54) Title: COMBINATION OF A SOLUBLE COMPLEMENT RECEPTOR -1(SCR1) AND AN AMIDINOPHENYL OR AMIDINO NAPHTHYL-ESTER FOR TREATING INFLAMMATION

(57) Abstract

A method of treating a disease or disorder associated with inflammation or inappropriate complement activation which method comprises administering to a mammal in need thereof an effective amount of a soluble CR1 protein and an effective amount of an amidinophenyl or amidinonaphthyl ester, including pharmaceutically acceptable salts thereof.

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COMBINATION OF A SOLUBLE COMPLEMENT RECEPTOR-1(SCR1) AND AN AMIDINOPHENYL OR AMIDINONAPHTYL ESTER FOR TREATING INFLAMMATION

The present invention relates to therapeutic compositions of protease inhibitors and human soluble complement receptor 1 which act synergistically to inhibit activation of complement. Such compositions are useful in the therapy of inflammatory or immune disorders involving complement activation.

Complement receptor type 1 (CR1) is present on the membranes of erythrocytes, monocytes/macrophages, granulocytes, B cells, some T cells, splenic follicular dendritic cells, and glomerular podocytes. CR1 binds to the complement components C3b and C4b and has also been referred to as the C3b/C4b receptor. The structural organisation and primary sequence of one allotype of CR1 is known (Klickstein *et al.*, 1987, J. Exp. Med. 165:1095-1112, Klickstein *et al.*, 1988, J. Exp. Med. 168:1699-1717; Hourcade *et al.*,1988, J. Exp. Med. 168:1255-1270, WO 89/09220, WO 91/05047). It is composed of 30 short consensus repeats (SCRs) that each contain around 60-70 amino acids. In each SCR, around 29 of the average 65 amino acids are conserved. Each SCR has been proposed to form a three dimensional triple loop structure through disulphide linkages with the third and first and the fourth and second half-cystines in disulphide bonds. CR1 is further arranged as 4 long homologous repeats (LHRs) of 7 SCRs each. Following a leader sequence, the CR1 molecule consists of the N-terminal LHR-A, the next two repeats, LHR-B and LHR-C, and the most C-terminal LHR-D followed by 2 additional SCRs, a 25 residue putative transmembrane region and a 43 residue cytoplasmic tail.

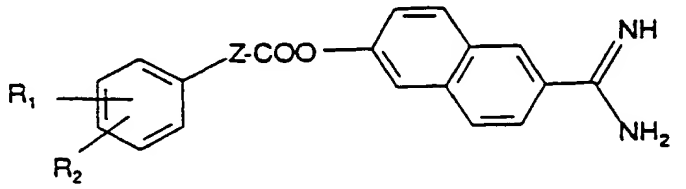
Several soluble fragments of CR1 have been generated via recombinant DNA procedures by eliminating the transmembrane region from the DNAs being expressed (WO 89/09220, WO 91/05047). The soluble CR1 fragments were functionally active, bound C3b and/or C4b and demonstrated Factor I cofactor activity depending upon the regions they contained. Such constructs inhibited *in vitro* complement-related functions such as neutrophil oxidative burst, complement mediated haemolysis, and C3a and C5a production. A particular soluble construct, sCR1/pBSCR1c, also demonstrated *in vivo* activity in a reversed passive Arthus reaction (WO 89/09220, WO 91/05047; Yeh *et al.*, 1991, J. Immunol. 146:250-256), suppressed post-ischemic myocardial inflammation and necrosis (WO 89/09220, WO 91/05047; Weisman *et al.*, Science, 1990, 249:146-151; Dupe, R. *et al.* Thrombosis & Haemostasis (1991) 65(6) 695.) and extended survival rates following transplantation (Pruitt & Bollinger, 1991, J. Surg. Res 50:350; Pruitt *et al.*, 1991 Transplantation 52; 868), as well as demonstrating therapeutic inhibition of complement activation in several animal models of disease such as lung injury (Rabinovici *et al*, 1992 J. Immunol. 149:1744-1750; Mulligan *et al*, 1992 J. Immunol. 148:3086-3092), intestinal ischaemia (Hill *et al*, 1992 FASEB J. 6:A1049) and acute

myocardial infarction (Weisman *et al*, 1990 Science 249:146-151, Dupe *et al*,1991 (above)).

In a number of cases, the doses of sCR1 required for therapeutic effects in these models were large (>5mg/kg). Because sCR1 is a biopharmaceutical produced by mammalian cell culture techniques, it is desirable to reduce the dose and hence the cost of therapy.

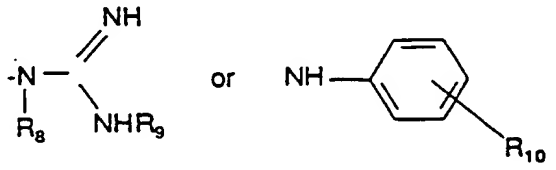
Certain amidinophenyl and amidinonaphthyl esters of carboxylic acids are known to be inhibitors of complement activation as well as having antitrypsin, antiplasmin, antikallikrein and antithrombin activity (GB 2095-239, GB 2083-818).

GB 2083818 discloses compounds of formula (A):



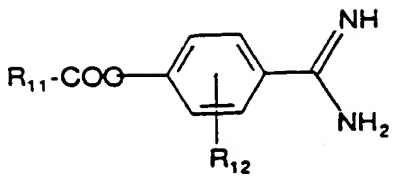
(A)

wherein Z represents $-(CH_2)_a-$, $-(CH_2)_b-CH(R_3)-$, $-CH=C(R_4)-$ or $-O-CH(R_4)-$, where a is 0, 1, 2 or 3, b is 0, 1 or 2, R_3 is a straight or branched chain alkyl group of 1 to 4 carbon atoms or a cycloalkyl group of 3 to 6 carbon atoms, and R_4 is a hydrogen atom or a straight or branched chain alkyl group of 1 to 4 carbon atoms and wherein the $-CH(R_3)-$, $=C(R_4)-$ or $-CH(R_4)-$ moiety is bonded to the -COO group; and R_1 and R_2, which may be the same or different, represent each a hydrogen atom, straight or branched chain alkyl group of 1 to 4 carbon atoms, $-O-R_5$, $-S-R_5$, $-COOR_5$, $-COR_6$, $-O-COR_7$, $-NHCOR_7$, $-(CH_2)_c-NR_8R_9$, $-SO_2NR_8R_9$, NO_2, CN, halogen, CF_3, methylenedioxy,



where c is 0, 1 or 2; R_5 is a hydrogen atom, straight or branched chain alkyl group of 1 to 4 carbon atoms, or benzyl group; R_6 is a hydrogen atom or straight or branched chain alkyl group of 1 to 4 atoms; R_7 is a straight or branched chain alkyl group of 1 to 4 carbon atoms; R_8 and R_9, which may be the same or different, are each a hydrogen atom, straight or branched chain alkyl group of 1 to 4 carbon atoms, or amino radical protecting group; and R_{10} is a hydrogen atom, dimethyl or CF_3.

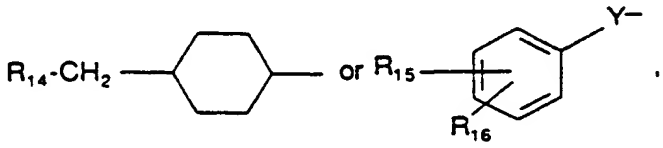
GB 2095239 discloses compounds of the general formula (B):



(B)

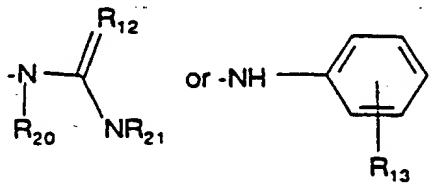
wherein

R_{11} represents a straight or branched chain alkyl group 1 to 6 carbon atoms, a straight or branched chain alkenyl group of 2 to 6 carbon atoms having 1 to 3 double bonds, R_{13}-$(CH_2)_d$-, R_{14}-$(CH_2)_e$-,



where R_{13} is a cycloalkyl group of 3 to 6 carbon atoms or a cycloalkenyl group of 3 to 6 atoms having 1 or 2 double bonds; d is 0, 1, 2 or 3; R_{14} is an amino or guanidino group or a protected amino or guanidino group; e is a number from 1 to 5; R_{15} and R_{16}, which may be the same or different, are each a hydrogen atom, a straight or branched alkyl group of 1 to 4 carbon atoms, -OR_{17}, methylenedioxy group, -SR_{17}, -$COOR_{17}$, -COR_{18}, -$OCOR_{19}$, -$NHCOR_{19}$, -$(CH_2)_f$-$NR_{20}R_{21}$ (f is 0, 1,2),

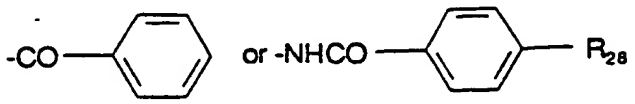
-$SO_2NR_{20}R_{21}$, a halogen atom, -CF_3, NO_2, CN,



R_{17} is a hydrogen atom, a straight or branched alkyl group of 1 to 4 carbon atoms or a benzyl group; R_{18} is a hydrogen atom, a straight or branched alkyl group of 1 to 4 carbon atoms; R_{19} is a straight or branched alkyl group of 1 to 4 carbon atoms; R_{20} and R_{21}, which may be the same or different, are each a hydrogen atom, a straight or branched alkyl group of 1 to 4 carbon atoms, or an amino-protecting group; R_{22} is O, S or NH; R_{23} is a 2',3'-dimethyl or 3'-CF_3 group; Y is -$(CH_2)_g$-(g is 0, 1, 2 or 3), -$(CH_2)_h$-CHR_{24}- (h is 0, 1 or 2), or -CH=CR_{25}-;

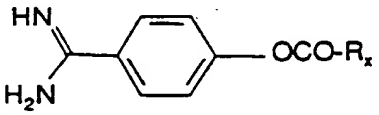
R_{24} is a straight or branched alkyl group of 1 to 4 carbon atoms and the carbon atom or the CHR_{24} moiety is attached to the COO group; R_{25} is a hydrogen atom or a straight or branched alkyl group of 1 to 4 carbon atoms and the carbon

atom of the CR_{25} moiety is attached to the COO group; and R_{12} represents $-R_{26}$, $-OR_{26}$, $-COOR_{27}$, one or two of the same halogen atoms, $-NH_2$, $-SO_3H$,



wherein R_{26} is a straight or branched alkyl group of 1 to 4 carbon atoms; R_{27} is a hydrogen atom or a straight or branched alkyl group of 1 to 4 carbon atoms; and R_{28} is a hydrogen atom or a guanidino group.

Other amidinophenyl esters of carboxylic acids are also known to inhibit proteases of the coagulation pathway (A.D.Turner *et al*, 1986 Biochem. 25:4929-35) and have also been employed to acylate the active centres of fibrinolytic enzymes reversibly (US 4,285,932, US 4,507,283, EP 0,297,882, R.A.G.Smith *et al*, 1985 Progress in Fibrinolysis VII 227-231). US 4285932, US 4507283 and EP 0297882 disclose compounds of formula (C):



(C)

wherein R_X is

benzoyl optionally substituted with one or two substituents independently selected from halogen, C_{1-6} alkyl, C_{2-6} alkenyl, C_{1-6} alkoxy, C_{1-6} alkanoyloxy, C_{1-6} alkanoylamino, amino, dimethylamino or guanidino;

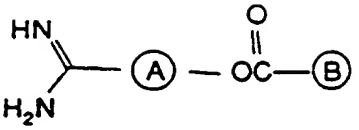
naphthoyl; or

acryloyl optionally substituted with C_{1-6} alkyl, furyl or phenyl wherein the phenyl moiety is optionally substituted with C_{1-6} alkyl.

Thus this type of compound is not a specific inhibitor of the proteases of the complement system.

Synergistic compositions of CR1-related polypeptides with certain organic compounds have been described (W0 92/10205).

According to the present invention there is provided a method of treating a disease or disorder associated with inflammation or inappropriate complement activation which method comprises administering to a mammal in need thereof an effective amount of a soluble CR1 protein and an effective amount of an amidinophenyl or amidinonaphthyl ester of formula (I) having complement inhibitory activity:



(I)

wherein A is phenyl optionally substituted with C_{1-4} alkyl, C_{1-4} alkoxy, C_{1-4} alkoxycarbonyl, halo, NH_2, sulphonyl, benzoyl or C_{1-4} alkylbenzoylamino or naphthyl; and

B is CH_2=CH- optionally substituted by a group selected from C_{1-6} alkyl, phenyl and phenyl substituted with C_{1-6} alkyl; phenyl optionally substituted with one or two substituents independently selected from halogen, C_{1-6} alkyl, C_{2-6} alkenyl, C_{1-6} alkoxy, C_{1-6} alkenoyloxy, C_{1-6} alkanoylamino, amino, dimethylamino or guanidino; or naphthyl, including pharmaceutically acceptable salts thereof.

Preferably, A is phenyl optionally substituted in the 2- or 3- position by halogen and the amidine substituent is in the 4-position of the phenyl ring. B is preferably phenyl 4-substituted by C_{1-4} alkoxy and optionally further substituted by halogen. Most preferably, B is 4-methoxyphenyl and A is phenyl or 2-bromophenyl, 4-substituted by the amidine group.

Suitable examples of halo include chloro and bromo.

Pharmaceutically acceptable salts may be formed with pharmaceutically acceptable acids, for example, maleic, hydrochloric, hydrobromic, phosphoric, acetic, fumaric, salicylic, citric, lactic, mandelic, tartaric, methanesulphonic and oxalic acid.

In a preferred aspect, the soluble CR1 component used in combination therapy is encoded by a nucleic acid vector selected from the group consisting of pBSCR1c, pBSCR1s, pBM-CR1c, pBSCR1c/pTCSgpt and pBSCR1s/pTCSgpt, and is especially that obtainable from pBSCR1c/pTCSgpt, as described in WO 89/09220.

The amounts of each compound are chosen such that the concentration of each component required to inhibit by 50% haemolysis of sensitized erythrocytes in a standard complement assay is lowered compared with that required for the individual components in the same assay. This increase in potency is described by a synergy factor which is defined in more detail below.

The invention also provides the use of a soluble CR1 protein and an amidinophenyl or amidinonaphthyl ester having complement inhibitory activity in the manufacture of a medicament for the treatment of a disease or disorder associated with inflammation or inappropriate complement activation.

The compounds may be administered by standard routes, such as, for example, intravenous infusion or bolus injection, and may be administered together or sequentially, in any order.

When the compounds are administered together they are preferably given in the form of a pharmaceutical composition comprising both agents. Thus, in a further

aspect of the invention there is provided a pharmaceutical composition comprising a soluble CR1 protein and an amidinophenyl or amidinonaphthyl ester having complement inhibitory activity together with a pharmaceutically acceptable carrier.

In a preferred embodiment, the composition may be formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings.

In a further aspect, the invention therefor provides a method for the preparation of a pharmaceutical composition of the invention, which method comprises admixing the combination of soluble CR1 protein and an amidinophenyl or amidinonaphthyl ester of formula (I), including pharmaceutically acceptable salts thereof.

The present invention also provides a method of treating a disease or disorder associated with inflammation or inappropriate complement activation comprising administering to a subject in need of such treatment a therapeutically effective amount of a composition of the invention.

In the above methods, the subject is preferably a human.

An effective amount of the protein for the treatment of a disease or disorder is in the dose range of 0.01-100mg/kg; preferably 0.1-10mg/kg.

An effective amount of the ester for the treatment of a disease or disorder is in the dose range 0.05-100 mg/kg; preferably 0.05-10 mg/kg. The ratio of protein to ester is preferably in the range 1:1 to 1:20 by weight.

The composition typically contains a therapeutically active amount of the protein and ester and a pharmaceutically acceptable excipient or carrier such as saline, buffered saline, dextrose, or water. Compositions may also comprise specific stabilising agents such as sugars, including mannose and mannitol, and local anaesthetics for injectable compositions, including, for example, lidocaine.

A pharmaceutical pack comprising one or more containers filled with one or more of the ingredients of the pharmaceutical composition is also within the scope of the invention.

The present invention also provides a method for treating a thrombotic condition, in particular acute myocardial infarction, in a human or non-human animal, said method comprising administering to the patient a composition according to this invention.

This invention further provides a method for treating adult respiratory distress syndrome (ARDS) in a human or non-human animal, said method comprises administering to the patient a composition according to this invention.

The invention also provides a method of delaying hyperacute allograft or hyperacute xenograft rejection in a human or non-human animal which receives a transplant by administering a composition according to this invention.

The methods and compositions of this invention are useful in the treatment of complement-mediated or complement-related disorders, including but not limited to those listed below.

Disease and Disorders Involving Complement

Neurological Disorders
multiple sclerosis
stroke
Guillain Barré Syndrome
traumatic brain injury
Parkinson's disease
allergic encephalitis

Disorders of Inappropriate or Undesirable Complement Activation
hemodialysis complications
hyperacute allograft rejection
corneal graft rejection
xenograft rejection
interleukin-2 induced toxicity during IL-2 therapy
paroxysmal nocturnal haemoglobinuria

Inflammatory Disorders
inflammation of autoimmune diseases
Crohn's Disease
adult respiratory distress syndrome
thermal injury including burns or frostbite
uveitis

Post-Ischemic Reperfusion Conditions
myocardial infarction
balloon angioplasty
post-pump syndrome in cardiopulmonary bypass or renal hemodialysis
renal ischemia
hepatic ischemia

Infectious Diseases or Sepsis
multiple organ failure
septic shock

Immune Complex Disorders and Autoimmune Diseases

rheumatoid arthritis

systemic lupus erythematosus (SLE)

SLE nephritis

proliferative nephritis

glomerulonephritis

hemolytic anemia

myasthenia gravis

Reproductive Disorders

antibody- or complement-mediated infertility

MATERIALS

BRL 55730 - is the soluble complement receptor type 1 derived from the expression of plasmid pBSCR1c/pTCSgpt in CHO cells (WO 89/09220).

BRL24894A (APAN) - 4-amidinophenyl 4'-methoxybenzoate HCl (EP-0009879)

BRAPAN - 4-amidino-2-bromophenyl 4'-methoxybenzoate HCl (Example 3).

METHODS

Anti-complement Activity Measured by the Haemolysis of Sheep Erythrocytes

Functional activity of complement inhibitors was assessed by measuring the inhibition of complement mediated lysis of sheep erythrocytes sensitised with rabbit antibodies (obtained from Diamedix Corporation, Miami, USA). Human serum diluted 1:125 or 1/35.7 in 0.1 M Hepes pH 7.4/ 0.15 M NaCl buffer was the source of complement and was prepared from a pool of volunteers essentially as described in Dacie & Lewis, 1975 (Practical Haematology 5th Edition, Churchill Livingstone, Edinburgh and New York, pp3-4). Briefly, blood was warmed to 37°C for 5 minutes, the clot removed and the remaining serum clarified by centrifugation. The serum fraction was split into small aliquots and stored at -196°C. Aliquots were thawed as required and diluted in the Hepes buffer immediately before use.

Inhibition of complement-mediated lysis of sensitised sheep erythrocytes was measured using a standard haemolytic assay using a v-bottom microtitre plate format as follows, essentially as described by Weisman *et al* 1990 (above).

Standard assay

25 µl of a range of concentrations of inhibitor (typically in the region of 0.1µg/ml - 0.00078µg/ml final for BRL55730 and 100 - 0.1µM final of APAN or BRAPAN) diluted in Hepes (0.1M Hepes pH7.4/0.15M NaCl) buffer were incubated with 25 µl of buffer and 50 µl of the 1/125 diluted serum for 15 minutes at 37°C. 100 µl of prewarmed sensitised sheep erythrocytes were added for 1 hour at 37°C in a final reaction volume of 200 µl . Samples were spun at 300g at 4°C for 15 minutes before transferring 150 µl of supernatant to flat bottom microtitre plates and determining the absorption at 410 nm, which reflects the amount of lysis in each test solution. Maximum lysis was determined by incubating serum with erythrocytes in the absence of any inhibitor (E+S) from which the proportion of background lysis had been subtracted (determined by incubating erythrocytes with buffer) (E). The background lysis by inhibitor was assessed by incubating inhibitor with erythrocytes (E+I) and then subtracting that from test samples (E+I+S). Inhibition was expressed as a fraction of the total cell lysis such that IH50 represents the concentration of inhibitor required to give 50% inhibition of lysis. For experiments in which serum had been diluted 1/35.7, the incubation time was reduced to 15 mins at 37°C. Otherwise conditions were the same.

Maximum Lysis: $A_{max} = (E+S) - (E)$

Lysis in presence of inhibitor: $A_o = (E+I+S) - (E+I)$

Amount of inhibition: $IH = \frac{A_{max} - A_o}{A_{max}}$

Plots were made of [inhibitor] vs IH and IH_{50} values were determined from the titration curve by reading off the concentration corresponding to IH=0.5.

Synergy Assays

The assay was carried out in a similar manner to that described above except that inhibitor 1 eg BRL55730 was titrated in the presence of a fixed concentration of inhibitor 2 eg APAN. This was carried out by adding 25 µl of inhibitor 1 to 25 µl of inhibitor 2 in the presence of serum and measuring the degree of lysis as described above.

Determination of the Synergy Factor

For each synergy experiment both inhibitors were titrated on their own as well as together.

EXAMPLE 1

Inhibitor 1, BRL55730 was titrated on its own and in the presence of various concentrations of inhibitor 2, APAN. A plot was made of the [BRL55730] vs IH with and without APAN (Fig.1). The IH50 of BRL55730 was estimated at each APAN concentration. A second plot of [APAN] vs IH was made (Fig.2) from which the IH corresponding to the concentration of APAN used in the synergy experiment was estimated. The results were then tabulated (Table 1).

Column 1 refers to the concentration of APAN. The proportion of inhibition that a particular concentration of APAN contributes was estimated from the plot of [APAN] vs IH (Fig.2) (column 2). The IH50 for BRL55730 was determined at each concentration of APAN (column 3). The contribution that a particular concentration of APAN made to the IH50 of BRL55730 was subtracted ie 0.5 - $IH_{(APAN)}$ (column 4). This value was used to read off the concentration of BRL55730 which alone would have provided this level of inhibition (column 5). The adjusted BRL55730 concentration was divided by the measured IH50 to give the synergy factor i.e. column 5/column 3 = column 6. If the effect of APAN was additive, the synergy factor would be 1; values greater than 1 represent a synergistic effect and the greater the value, the greater the degree of synergy.

Isobologram Analysis

The IH50's of inhibitor 1, eg BRL55730 and inhibitor 2, eg APAN were determined separately. These experimentally determined values are plotted on the axes of the isobologram and were connected by a straight line, termed the line of additivity (Fig. 3). This line represents combinations of the two inhibitors which, when used together, would result in 50% inhibition (Tallarida (1992) Pain 49: 93-97, Miaskowski & Levine, (1992) 51: 383-387). Hence points falling on the line of additivity indicate an additive effect, points above this line indicate antagonism and points below this curve indicate synergy. BRL55730 was titrated in the presence of fixed concentrations of APAN and the IH50 of BRL55730 determined at each APAN concentration. This data was plotted on the isobologram (Fig. 3).

Statistical Analysis of Synergy using Fixed Concentration Pairs.

BRL55730 at concentration x which was below its IH50 was assayed in the standard assay. APAN at concentration y which was below its IH50 was also assayed. Then $BRL55730_{[x]}$ was assayed together with $APAN_{[y]}$ in the same haemolysis assay. The amount of inhibition was calculated for the two inhibitors when assayed separately and when assayed together.

If synergy occurs then the inhibition of the compounds assayed together should be greater than the sum of the two inhibitors separately

ie $BRL55730_{[x]}/APAN_{[y]} > BRL55730_{[x]} + APAN_{[y]}$

The data was analysed statistically by t-test according to the formula listed below.

Group	Mean	Sample size	Standard error of the mean	Standard error of the mean squared
a BRL55730	a	n_a	$s.e.m._a$	$(s.e.m.)_a^2$
b APAN	b	n_b	$s.e.m._b$	$(s.e.m)_b^2$
ab BRL55730/APAN	ab	n_{ab}	$s.e.m_{ab}$	$(s.e.m.)_{ab}^2$

Null Hypothesis = H0 $ab = a + b$ (ie effect is additive)

Alternative Hypothesis = H1 $ab \neq a + b$ (ie effect is not additive)

$$t = \frac{ab - a - b}{\sqrt{\{(s.e.m._{ab}^2) + (s.e.m._a^2) + (s.e.m._b^2)\}}} \qquad \text{Equation 1}$$

t was compared with critical levels in t-tables where degrees of freedom (df)

$$df = n_a + n_b + n_{ab} - 3$$

a. **Synergy of APAN with BRL55730 in Serum Diluted 1/125**

BRL 24894A (APAN) molecular weight 324.24 was made 50 mM in dimethylsulphoxide (DMSO). BRL55730 (in 10mM sodium phosphate pH7.2 buffer) was at 5.3 mg/ml. Both inhibitors were titrated in the standard assay over the concentration range of 100 µM - 0.78 µM for APAN and 0.125 µg/ml - 0.00098 µg/ml for BRL55730. Two titration curves were performed for BRL55730 from which the mean IH50 was determined as 0.01 µg/ml (Fig.1) and one curve for APAN from which the IH50 was determined as 10 µM (Fig.2). To test for synergy, BRL55730 was titrated over the same concentration range but in the presence of fixed concentrations of APAN from 1 - 6 µM for each titration (Fig.1). From the data the synergy factor was calculated as described above.

Table 1: Determination of the Synergy Factor for BRL55730 and APAN.

1	2	3	4	5	6
[APAN] μM	IH of APAN	IH50 of BRL55730 μg/ml	0.5 - IH of APAN	Adjusted [BRL55730] μg/ml	Synergy Factor
0	-	0.01	0.5	0.01	1
1	0.03	0.007	0.47	0.009	1.3
2	0.09	0.004	0.41	0.008	2.0
3	0.16	0.0027	0.34	0.006	2.2
4	0.21	0.0022	0.29	0.004	1.8
5	0.27	0.0019	0.23	0.003	1.6
6	0.31	0.0013	0.19	0.0022	1.7

From the data in Table 1, inclusion of 6 μM APAN with BRL55730 reduces the IH50 by approximately 8 fold. The calculated synergy factor at each concentration of APAN is given in Table 1 and shows that the effect of APAN is more than additive since the synergy factor is > 1. The synergy factor also remains fairly constant across the range of concentrations used with a mean value of 1.8.

BRL55730, concentration range 0.04 - 0.000039 μg/ml, was titrated on its own; the concentration at which no inhibition of complement activation occurred was found to be ~ 0.0004 μg/ml. Titration of APAN on its own showed that a concentration of 4 μM gave an IH of 0.31 and 2 μM gave an IH of 0.16. When BRL55730 was titrated in the presence of APAN at 4 and 2 μM, the inhibition at 0.0004 μg/ml of BRL55730 was greater than that could be accounted for by APAN only showing that APAN potentiates the activity of BRL55730 below the no-effect concentration.

b. Isobologram Analysis BRL55730 with APAN

The additivity line was constructed as described above taking the data from Figs. 1 & 2. The IH50's of BRL55730 at each APAN concentration (columns 1 & 3 of Table 1 respectively) were then plotted on the isobologram as shown in Fig. 3. The points fall below the line of additivity indicating that the interaction is synergistic.

c. **Statistical Analysis of Synergy Between BRL55730 and APAN using Fixed Concentration Pairs**

The following concentration pairs were tested for synergy as described above

(i) 0.005 µg/ml BRL55730 4 µM APAN
(ii) 0.005 µg/ml BRL55730 2 µM APAN
(iii) 0.002 µg/ml BRL55730 4 µM APAN
(iv) 0.002 µg/ml BRL55730 2 µM APAN

The statistical parameters are given below for each concentration pair.

Table 2: Statistical Analysis of Concentration Pair (i)

PARAMETER	BRL55730 0.005 µg/ml	APAN 4 µM	BRL55730 + APAN 0.005 µg/ml + 4 µM
MEAN IH	0.312	0.297	0.676
SEM	0.0117	0.0092	0.0096
$(SEM)^2$	0.000136	0.000086	0.000092
n	14	14	14
df	39		
t-value	3.822		
	At 39 df, probablility of 0.9995 t = 3.558 Calculated t > 3.558. Therefore a + b ≠ ab		

Table 3: Statistical Analysis of Concentration Pair (ii)

PARAMETER	BRL55730 0.005 µg/ml	APAN 2 µM	BRL55730 + APAN 0.005 µg/ml + 2 µM
MEAN IH	0.251	0.124	0.509
SEM	0.0135	0.0183	0.00947
$(SEM)^2$	0.000183	0.000334	0.0000897
n	16	16	16
df	45		
t-value	5.407		
	At 45 df, probablility of 0.9995 t = 3.550 Calculated t > 3.550. Therefore a + b ≠ ab		

Table 4: Statistical Analysis of Concentration Pair (iii)

PARAMETER	BRL55730 0.002 µg/ml	APAN 4 µM	BRL55730 + APAN 0.002 µg/ml + 4 µM
MEAN IH	0.123	0.337	0.564
SEM	0.00741	0.00972	0.00691
$(SEM)^2$	0.000055	0.0000945	0.0000477
n	16	16	16
df	45		
t-value	7.451		
	At 45 df, probablility of 0.9995 t = 3.550 Calculated t > 3.550. Therefore a + b ≠ ab		

Table 5: Statistical Analysis of Concentration Pair (iv)

PARAMETER	BRL55730 0.002 µg/ml	APAN 2 µM	BRL55730 + APAN 0.002 µg/ml + 2 µM
MEAN IH	0.121	0.192	0.422
SEM	0.0104	0.0106	0.00886
$(SEM)^2$	0.000109	0.000112	0.0000784
n	16	16	16
df	45		
t-value	6.267		
	At 45 df, probablility of 0.9995 t = 3.550 Calculated t > 3.550. Therefore a + b ≠ ab		

At each of the tested concentration pairs, the alternative hypothesis was shown to be correct ie that the effect was not additive and since a + b < ab, synergy has been demonstrated.

d. Synergy Effect of BRL55730 on APAN

The effect of BRL55730 on the IH50 of APAN was tested in the same way as described in Example 1a but in this instance APAN was titrated over the range 0.78µM to 100µM in the presence of fixed concentrations of BRL55730 between 0.001 - 0.006 µg/ml. The effect of BRL55730 on the IH50 of APAN is given in Table 2 and shows that addition of 0.006 µg/ml of BRL55730 shifts the IH50 of APAN from 10 µM to 1 µM which is an improvement of 10 fold. The synergy factor was determined as described in the Methods and found to be > 1 (Table 6)

indicating that the synergy process between APAN and BRL55730 is reversible. Unlike the previously described Example 1a, the synergy factor appears to be dependent on the concentration of BRL55730.

Table 6: Determination of the Synergy Factor for APAN and BRL55730

[BRL55730] µg/ml	IH of BRL55730	IH50 of APAN µM	0.5 - IH of BRL55730	Adjusted [APAN] µM	Synergy Factor
0	-	10	0.5	10	1
0.001	0.025	5.3	0.475	9	1.7
0.003	0.13	1.8	0.37	7	3.9
0.006	0.34	1.0	0.16	3	3.0

e. Isobologram Analysis of APAN with BRL55730

Isobologram analysis was performed as described above using data from Fig. 2 and Table 6. Data points fell below the line of additivity which indicated the effect was synergistic.

f. Synergy of APAN with BRL55730 in Serum Diluted 1/35.7

To test whether the synergy seen between APAN and BRL55730 is reproducible in more concentrated serum, experiments were carried out in serum that had been diluted 1/35.7 which was 3.5 fold more concentrated than described in Example 1a. As a preliminary test to make sure that this concentration of serum did not produce maximum lysis of the sensitised sheep erythrocytes, 50 µl of serum at various dilutions from 1/10 to 1/150 were preincubated with 50 µl of 0.1 M Hepes pH 7.4 / 0.15 M NaCl buffer at 37°C for 15 mins. 100 µl of erythrocytes were added and samples were incubated for either 15 mins or 30 mins at 37°C. Unlysed cells were spun down at ~ 300 g at 4°C for 15 mins and then 150 µl of supernatant were transfered to a flat bottom microtitre plate before reading the absorbance at 410 nm. A plot of the serum dilution vs absorbance showed that incubation of serum at 1/35.7 dilution for 15 mins with erythrocytes gave ~ 90% of the maximum lysis. Since this concentration of serum proved suitable, synergy experiments of BRL55730 with APAN were carried out in a similar manner as described in Example 1a except that more concentrated serum was used and incubation times were reduced to 15 mins.

Titration of BRL55730 in the more concentrated serum gave an IH50 of ~ 0.14 µg/ml (mean of two determinations) which is 14 fold greater than in 1/125

diluted serum. Similarly the IH50 of APAN was found to be 52 μM (mean of two determinations) which is about five fold greater than in the more dilute serum. The synergy experiments were carried out by titrating BRL55730 over a concentration range of 1 - 0.0078 μg/ml in the presence of APAN at concentrations ranging from 2 - 18 μM. The summary of the data is given in Table 7 and demonstrates that the synergy effect can be extended to more concentrated serum. The synergy factor in this instance appears to be dependent on the concentration of APAN.

Table 7: Synergy Value Between BRL55730 and APAN in more concentrated Serum

1	2	3	4	5	6
[APAN] μM	IH of APAN	IH50 of BRL55730 μg/ml	0.5 - IH of APAN	Adjusted [BRL55730] μg/ml	Synergy Factor
0	-	0.14	0.5	0.14	1
2	0	0.135	0.5	0.14	1.03
4	0.025	0.11	0.475	0.135	1.23
6	0.035	0.055	0.465	0.125	2.27
9	0.055	0.045	0.445	0.11	2.44
12	0.100	0.032	0.4	0.105	3.28
15	0.16	0.025	0.34	0.1	4.00
18	0.2	0.02	0.3	0.085	4.25

g. Isobologram Analysis of BRL55730 and APAN in More Concentrated Serum

The IH50's of BRL55730 and APAN in serum diluted 1/35.7 were used to construct the additivity line. Data points from columns 1 and 3 of Table 7 were used to construct an isobologram. All the points except at 2 μM fall below the addivitity line indicating synergy. The 2 μM data point shows a synergy factor of 1.03 in Table 7 and falls on the line of additivity in the isobologram showing that at this concentration the effect may be additive.

h. Synergy Effect of BRAPAN on BRL55730

4-Amidino-2-bromophenyl 4'-methoxybenzoate HCl (BRAPAN) molecular weight 386 was made 10 mM in DMSO and titrated as described in the Methods using serum diluted 1/125. From two separate determinations the mean value for the

IH50 of BRAPAN was 3 μM. A single titration curve of BRL55730 from 0.1 - 0.00078 μg/ml was determined which gave an IH50 of 0.021 μg/ml. To test for synergy BRL55730 was titrated over the same concentration range but in the presence of BRAPAN ranging from 0.1 μM to 0.9μM. Table 8 demonstrates the effect and synergy potential of BRAPAN on BRL55730. As with APAN at the same serum dilution the synergy factor is >1 indicating that BRAPAN synergises with the BRL55730. The synergy factor remains fairly constant over the concentration range giving a mean value of 1.7 which again is very similar to that seen for APAN.

Table 8: Synergy Value Between BRL55730 and BRAPAN

1	2	3	4	5	6
[BRAPAN] μM	IH of BRAPAN	IH50 of BRL55730 μg/ml	0.5 - IH of BRAPAN	Adjusted [BRL55730] μg/ml	Synergy Factor
0	0	0.021	0.5	0.021	1
0.1	0	0.015	0.5	0.021	1.4
0.2	0	0.013	0.5	0.021	1.62
0.3	0.01	0.011	0.49	0.02	1.82
0.4	0.03	0.013	0.47	0.019	1.46
0.5	0.04	0.012	0.46	0.018	1.5
0.6	0.06	0.008	0.44	0.016	2.00
0.7	0.09	0.007	0.41	0.015	2.14
0.8	0.13	0.007	0.37	0.013	1.86
0.9	0.16	0.0059	0.34	0.011	1.86

i. Isobologram Analysis of the effect of BRAPAN on BRL55730

Using the data described in Example 1h for BRAPAN, an isobologram was constructed. All the data points fell below the line of additivity indicating synergy was occurring.

EXAMPLE 2

Co-formulation of sCR1 (BRL 55730) and APAN (BRL 24894A)

D-Mannitol (Sigma.UK, 60mg) was dissolved in water for injection (9.5ml). APAN was disssolved in HPLC-grade methanol to a final concentration of 6 mg/ml by stiring at ambient temperature (20-25°C) for 5 min. The solution (0.5ml) was added IMMEDIATELY to the mannitol solution and mixed by shaking. A solution of

BRL 55730 (5mg/ml in 10mM sodium phosphate pH 7.2, 0.2ml) was added, shaken and immediately frozen in solid CO_2. The material was lyophilised at an average pressure of 2-3 mbar and a condenser temperature of -60°C for 20 hours. The white solid had the following composition and was stored desiccated at -70°C:
BRL 55730: 1mg; BRL 24894A: 3mg; D-Mannitol: 60mg; sodium phosphate: trace.

EXAMPLE 3

Preparation of 4-Amidino 2-bromophenyl 4'-methoxybenzoate HCl (BRAPAN)

This material was prepared in two steps from 2-bromo 4-cyanophenol.

a: Preparation of 2-bromo-4-amidinophenol hydrochloride

2-Bromo-4-cyanophenol (1.35 g, 6.8 mmole) was dissolved in ethanol (20 ml). Hydrogen chloride gas was passed through the cooled solution, a white precipitate forming after 45 mins. After 2 hours, the bubbling was stopped and the solid in solution placed at 4°C for two days. The white solid was isolated by filtration. This was rapidly suspended in ethanol solution (50 ml) and a saturated solution of ammonia in ethanol (75 ml) was added. The suspension went clear almost immediately and was stirred for 24 hours and allowed to stand for a similar period. All volatile material was removed and the white solid was taken up in water (20 ml). Addition of concentrated hydrochloric acid (5 ml) led to the rapid formation of a white crystalline mass which was isolated by filtration and recrystallised from ethanol (20 ml) and diethyl ether (100 ml). Yield: 860 mg (50%). mp: 279-80°.

^1H nmr ($CDCl_3$-d^6DMSO) δ: 9.2 (4H, br d, amidine), 8.2 (1H, d, J=2Hz, aryl-H), 7.8 (1H, m, aryl-H), 7.25 (1H, J=9Hz, aryl-H)
Infrared (nujol): 3325, 3125, 2300-3400, 1670, 1610, 1585, 1410, 1300, 1175, 1045, 880, 835, 725, 620 cm^{-1}
Analysis: C 33.45, H 3.28, N 10.95%
$C_7H_8N_2OBrCl$ requires C 33.43, H 3.21, N 11.14%

b. Preparation of the title compound

To a solution of 4-methoxybenzoyl chloride (271 mg, 1.59 mmole) in dry pyridine (5 ml) was added 2-bromo-4-amidinophenol hydrochloride (400 mg, 1.59 mmole). The initial suspension became a clear solution and then a precipitate reformed. After 1 hour stirring infrared analysis showed the formation of an ester. The pyridine was removed at reduced pressure the last traces by azeotrope with ethanol. The white solid obtained was recrystallised twice from 5:1 diethyl ether/ethanol (~ 100 ml) to leave the white title compound. Yield: 225 mg (37%). mp 212-3°C.

^1H nmr (d^6DMSO-trace $CDCl_3$) δ: 9.55 (4H, br, amidinophenol NH), 6.95-8.25 (7H, m, aryl-H), 3.9 (1H, s, OCH_3)

Infrared (nujol): 2500-3400, 1735, 1665, 1605, 1580,1260, 1230, 1170, 1070, 1020, and 830 cm^{-1}

Analysis: C 46.66, H 3.74, N 7.19%

$C_{15}H_{12}N_2BrCl$ requires C 46.72%, H 3.66%, N 7.26%

In the figures:

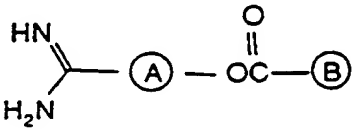
Fig. 1 shows the effect of different concentrations of APAN on BRL 55730;

Fig. 2 shows the inhibition of complement activation by APAN; and

Fig. 3 is an isobologram of BRL 55730 and APAN in a standard assay.

CLAIMS

1. A method of treating a disease or disorder associated with inflammation or inappropriate complement activation which method comprises administering to a mammal in need thereof an effective amount of a soluble CR1 protein and an effective amount of an amidinophenyl or amidinonaphthyl ester of formula (I) having complement inhibitory activity:



(I)

wherein A is phenyl optionally substituted with C_{1-4} alkyl, C_{1-4} alkoxy, C_{1-4} alkoxycarbonyl, halo, NH_2, sulphonyl, benzoyl or C_{1-4} alkylbenzoylamino or naphthyl; and

B is CH_2=CH- optionally substituted by a group selected from C_{1-6} alkyl, phenyl and phenyl substituted with C_{1-6} alkyl; phenyl optionally substituted with one or two substituents independently selected from halogen, C_{1-6} alkyl, C_{2-6} alkenyl, C_{1-6} alkoxy, C_{1-6} alkenoyloxy, C_{1-6} alkanoylamino, amino, dimethylamino or guanidino; or naphthyl, including pharmaceutically acceptable salts thereof.

2. The use of a soluble CR1 protein and an amidinophenyl or amidinonaphthyl ester of formula (I) as defined in claim 1 having complement inhibitory activity in the manufacture of a medicament for the treatment of a disease or disorder associated with inflammation or inappropriate complement activation.

3. A pharmaceutical composition comprising a soluble CR1 protein and an amidinophenyl or amidinonaphthyl ester of formula (I) as defined in claim 1 having complement inhibitory activity together with a pharmaceutically acceptable carrier.

4. A method of treating a disease or disorder associated with inflammation or inappropriate complement activation comprising administering to a subject in need of such treatment a therapeutically effective amount of a composition of claim 3.

5. A pharmaceutical pack comprising one or more containers filled with one or more of the ingredients of the pharmaceutical composition of claim 3.

6. A method for the preparation of a pharmaceutical composition according to claim 3, which method comprises admixing the combination of soluble CR1 protein and an amidinophenyl or amidinonaphthyl ester of formula (I) as defined in claim 1.

7. A method, use, composition, method, pack or method according to claim 1, 2, 3, 4, 5 or 6, respectively, wherein the soluble CR1 protein is that encoded by the nucleic acid vector pBSCR1c/pTCSgpt and the ester is 4-amidinophenyl 4'-methoxybenzoate HCl or 4-amidino-2-bromophenyl 4'-methoxybenzoate HCl.

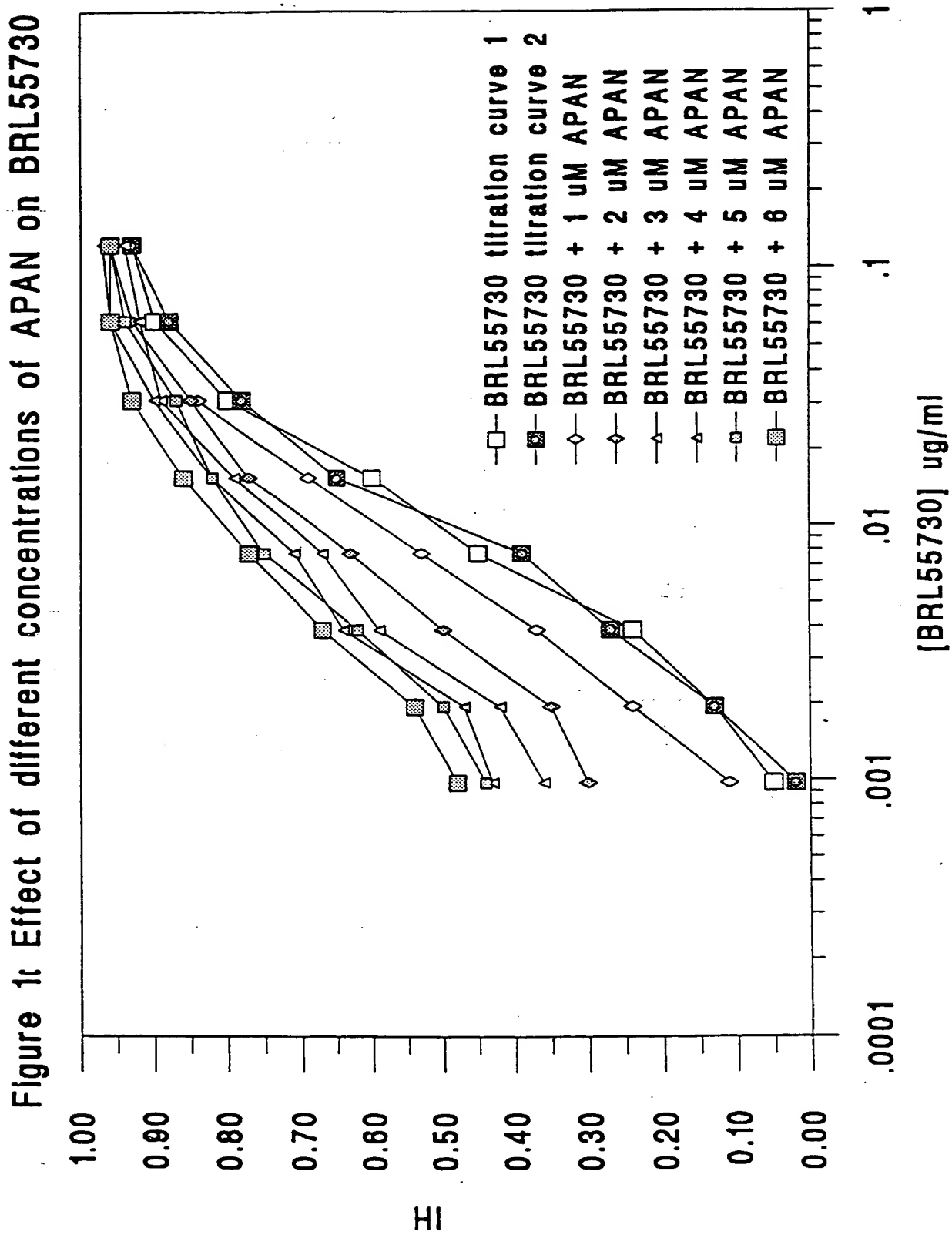


Figure 1: Effect of different concentrations of APAN on BRL55730
BRL55730 titration curve 1
BRL55730 titration curve 2
BRL55730 + 1 uM APAN
BRL55730 + 2 uM APAN
BRL55730 + 3 uM APAN
BRL55730 + 4 uM APAN
BRL55730 + 5 uM APAN
BRL55730 + 8 uM APAN
HI
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
.0001
.001
.01
.1
1
[BRL55730] ug/ml

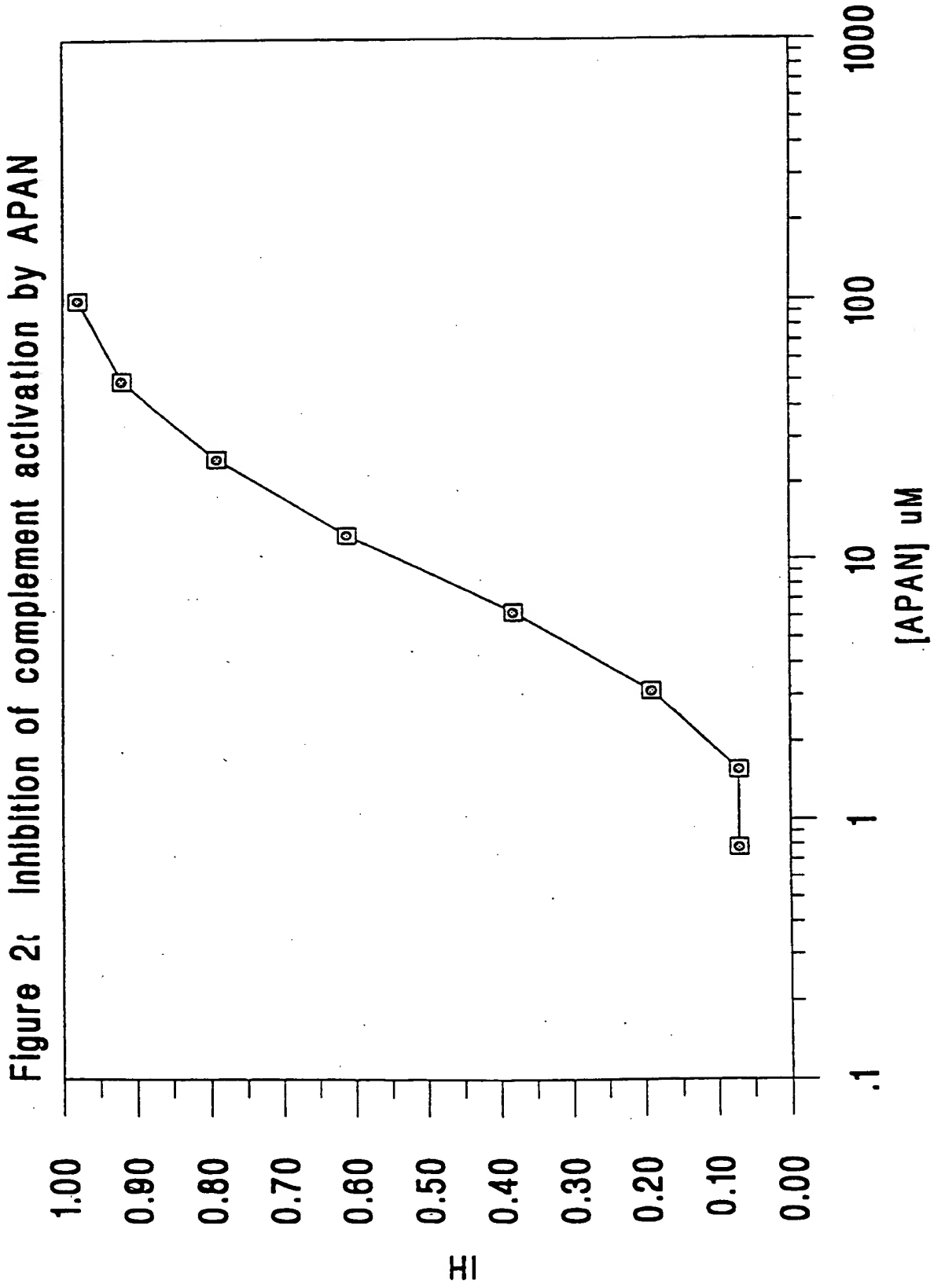


Figure 2: Inhibition of complement activation by APAN
HI
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
.1
1
10
100
1000
[APAN] uM

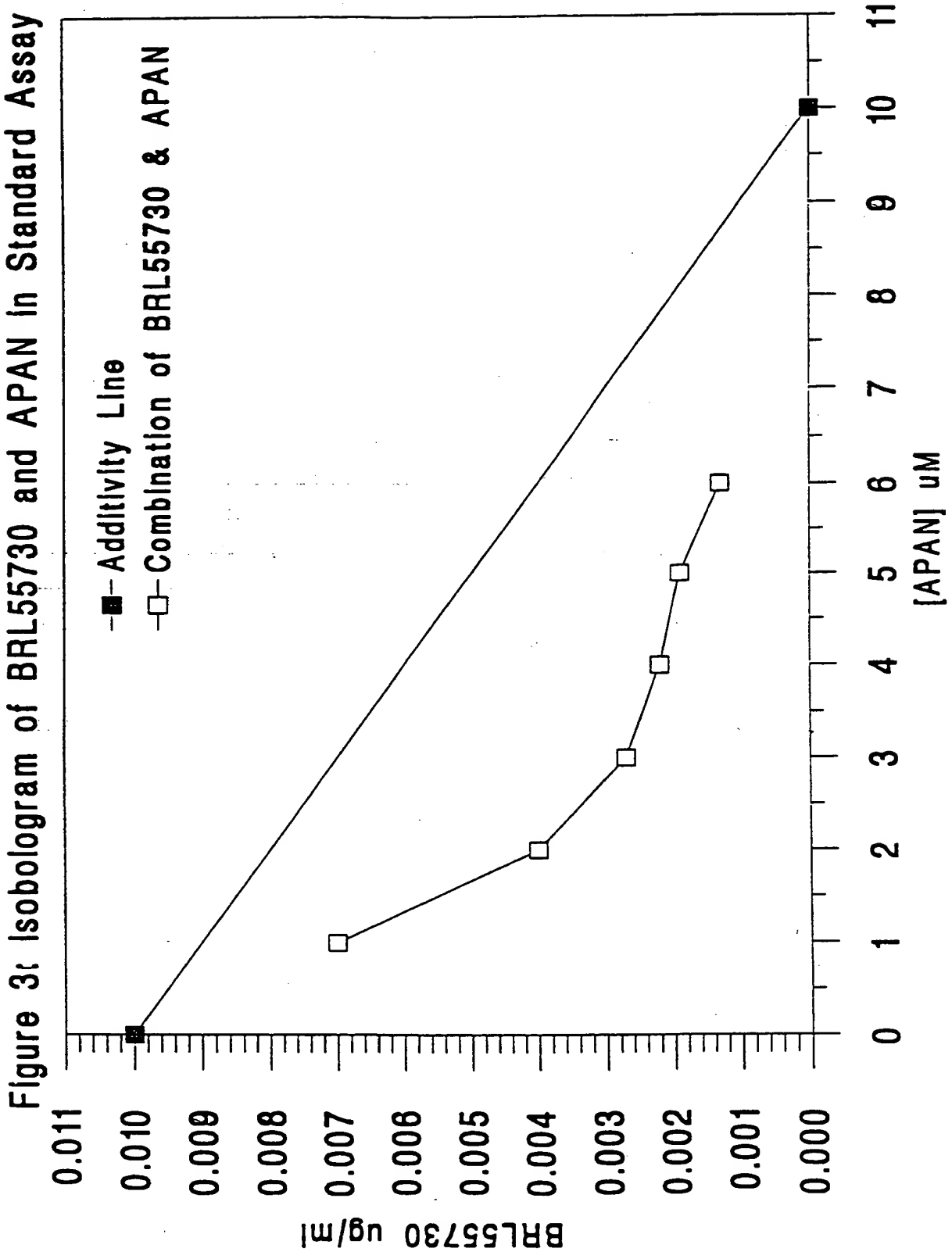


Figure 3: Isobologram of BRL55730 and APAN in Standard Assay
Additivity Line
Combination of BRL55730 & APAN
BRL55730 ug/ml
0.011
0.010
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
[APAN] uM
0
1
2
3
4
5
6
7
8
9
10
11

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/00122

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K37/02 A61K31/235

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 05047 (THE JOHNS HOPKINS UNIVERSITY) 18 April 1991 cited in the application see page 38 - page 46 see page 135; claims 113-121	1-7
Y	SCIENCE vol. 249, no. 4965 , 1990 pages 146 - 151 H.F. WEISMAN 'Soluble human complement receptor type 1: In vivo inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis.' cited in the application see the whole document	1-7

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 June 1994	04. 07. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Orviz Diaz, P

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CLIN. EXP. IMMUNOL. vol. 86, no. SUP1 , October 1991 pages 43 - 46 D.T. FEARON 'Anti-inflammatory and immunosuppressive effects of recombinant soluble complement receptors.' see the whole document	1-7
Y	J. AM. SOC. NEPHROL. (JASN) vol. 3, no. 3 , 1992 page 581 W. COUSER 'Soluble CR1 (BRL 55730, sCR1) suppresses glomerular injury in three types of complement-dependent glomerulonephritis' see abstract	1-7
Y	WO,A,92 10205 (T CELL SCIENCES, INC) 25 June 1992 cited in the application see claims	1-7
Y	EP,A,0 009 879 (BEECHAM GROUP LTD) 16 April 1980 cited in the application see the whole document, especially page 18, example 3	1-7
Y	GB,A,2 098 983 (TORII AND CO. LTD.) 1 December 1982 see abstract; claims see page 4; table 2	1-7
Y	GB,A,2 095 239 (TORII AND CO. LTD.) 29 September 1982 cited in the application see abstract; claims see page 9-15; table 2	1-7
Y	CHEM. PHARM. BULL. vol. 33, no. 4 , 1985 pages 1458 - 1471 T. AOYAMA 'Synthesis and structure-activity study of protease inhibitors. IV.Amidinonaphthols and related acyl derivatives.' see the whole document	1-7

2

INTERNATIONAL SEARCH REPORT

.ternational application No.
PCT/GB94/00122

Box I Observations where certain claims were f und unsearchable (Continuation of item 1 of first sheet)

This internati nal search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1,4 and 7 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compositions.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

B x II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inten nal Application No
PCT/GB 94/00122

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9105047	18-04-91	CA-A-	2067744	27-03-91
		CN-A-	1053265	24-07-91
		EP-A-	0502892	16-09-92
		JP-T-	5504053	01-07-93
		US-A-	5256642	26-10-93
WO-A-9210205	25-06-92	AU-A-	9149791	08-07-92
		EP-A-	0560929	22-09-93
		JP-T-	6503344	14-04-94
EP-A-0009879	16-04-80	AU-A-	5004679	13-03-80
		JP-A-	2154687	14-06-90
		US-A-	4285932	25-08-81
		US-A-	4337244	29-06-82
GB-A-2098983	01-12-82	JP-C-	1645261	28-02-92
		JP-B-	2059145	11-12-90
		JP-A-	57179147	04-11-82
		DE-A,C	3215676	11-11-82
		FR-A,B	2504526	29-10-82
		US-A-	4496584	29-01-85
GB-A-2095239	29-09-82	JP-C-	1509451	26-07-89
		JP-A-	57142957	03-09-82
		JP-B-	63060739	25-11-88
		JP-C-	1585337	31-10-90
		JP-B-	2010823	09-03-90
		JP-A-	58041855	11-03-83
		DE-A,C	3207033	30-09-82
		FR-A,B	2500825	03-09-82
		US-A-	4514416	30-04-85
		US-A-	4570006	11-02-86